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PRINCIPAL INVESTIGATOR: J. Kevin Foskett, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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J. Kevin Foskett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-Mail: foskett@mail.med.upenn.edu

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FOREWORD

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INTRODUCTION

Apoptosis, the morphological and biochemical manifestation of programmed cell death, plays a critical role in maintaining homeostasis of tissue and organ cell number, and is involved in differentiation, growth and development (1-4). Mammary gland physiology is strongly influenced by apoptosis in both normal and pathologic states. Involution of the lactating gland is due to apoptosis of differentiated epithelial cells, and an emerging hypothesis is that dysfunction of the apoptotic pathways in mammary gland is significantly involved in the causes and progression of breast cancer (5-9). Thus, definition of the biochemical pathways involved in mammary gland apoptosis is an important goal in breast cancer research. An important regulator of apoptosis is the bcl-2 oncogene (2,5). Bcl-2 expression prevents apoptosis in several cell types and is associated with a poor prognosis in response to various cancer therapies in patients. Bcl-2 is normally expressed at high levels in some tissues, including mammary gland. More recently, other bcl-2-related genes have been identified, defining a gene family. Like bcl-2, some are anti-apoptotic, whereas others promote apoptosis. It is likely that the pro:anti -apoptotic expression level ratio regulates sensitivity to apoptosis. Breast cancer is associated with an altered ratio, which correlates with failure to respond to therapy and poor survival (7-9). Thus, many breast cancers may be diseases of apoptosis. The molecular mechanisms which link bcl proteins to apoptosis are undefined, although bcl proteins act at a critical juncture which integrates different death signals and activates a single death pathway. Intracellular [Ca^{2+}] and intracellular Ca^{2+} stores may be involved in regulating apoptosis, and expression of bcl-2 has been linked to alterations in Ca^{2+} signaling and in the handling of Ca^{2+} by intracellular stores, including the endoplasmic reticulum (ER) and mitochondria (4,10-13). The bcl proteins are localized to the outer mitochondrial membrane, ER membrane and outer membrane of the nuclear envelope. Recent studies suggest that bcl-related proteins are closely associated with permeability pathways in membranes. Cyotchrome c (CytC) release from mitochondria *in vitro* could be blocked by bcl-2. In addition, bcl-xL was demonstrated to form ion channels in artificial membranes. These data suggest that bcl proteins can form and/or regulate channels, perhaps for organic (e.g. CytC) and well as for inorganic (e.g. Ca^{2+}) molecules. Nevertheless, the physiological relevance of these data are questionable without measurements of channel activity in the membranes in which these proteins normally reside in cells. This has not been possible because the intracellular location of the membranes has prevented use of rigorous electrophysiological approaches, in particular the single-channel patch clamp technique. My laboratory recently developed novel technology for measuring ER- and nuclear envelope-localized ion channel activities (14,15). We proposed to employ this approach, together with recombinant bcl proteins, stably-expressing cell lines and expression systems, in a novel series of experiments designed to determine whether bcl-related proteins form ion channels in the ER and nuclear envelope, and whether these proteins regulate the activities and regulation of other permeability pathways which exist in these membranes. The specific aims are to: 1. Determine whether recombinant bcl-related proteins can form functional ion channels in the outer membrane of the nuclear envelope; 2. Determine whether expression of bcl-related proteins confers novel ion channel activities in the outer nuclear membrane; 3. Determine the role of bcl-like proteins in influencing the activities of resident ion channels in the nuclear envelope and the permeability of the nuclear pore. These studies may provide direct evidence for a biochemical function of proteins critically involved in apoptosis, mammary gland biology and breast cancer.

BODY

Accomplishments during the third year

During the third year, we had difficulties replacing staff (Mr Sean McBride) to perform the electrophysiological analyses. This is a highly technical skill, and finding qualified individuals has been extremely difficult. We now have replacement personnel in the lab (Ms. Hayley Hormuth) that we have trained

in the art and science of nuclear patch clamping and preparation. We therefore did not spend the allocated monies during the last year, and consequently requested a no-cost extension of the project for one year. We intend to perform during the extension year the studies we proposed in our last annual report. For administrative purposes, I have repeated below what I summarized and proposed during the last report.

We proposed to undertake 3 specific aims during the 3 year granting period:

1. Determine whether recombinant bcl-related proteins can form functional ion channels in the outer membrane of the nuclear envelope.

2. Determine whether expression of bcl-related proteins confers novel ion channel activities in the outer nuclear membrane.

3. Determine the role of bcl-like proteins in influencing the activities of resident ion channels in the nuclear envelope and the permeability of the nuclear pore.

Our efforts during the first year had as their focus specific aim 2, with some attention also directed to specific aim 3. As proposed, we initiated experiments to determine whether heterologous expression of bcl-related proteins would result in novel ion channel activities in the outer membrane of the nuclear envelope. Our focus thus far has been on the *Xenopus* oocyte system, because of our familiarity with the procedures involved in the isolation of intact nuclei, patch clamp electrophysiology of the outer membrane, and expression of recombinant ion channels in this system. Because the *Xenopus* oocyte can express recombinant proteins, we reasoned that bcl-related proteins could be expressed and localized to the nuclear envelope, as in mammalian cells, and that patch clamp of the isolated nucleus could provide an opportunity to record ion channel activities which they might possess. Nevertheless, we did not detect novel channel activities,as we reported in our previous progress statement. We therefore refined our focus in two areas.

a. Development of a mammalian system for nuclear patch clamping. First, we considered that the oocyte expression system may not be optimal for the expression of recombinant mammalian proteins. We have therefore developed a comparable mammalian expression system that would enable patch clamp electrophysiology to be performed on isolated nuclei. To develop this system, we used Cos7 cells that had been transiently transfected with the rat type 1 inositol trisphosphate receptor. We have been able to routinely attain giga-ohn electrical seals on the nuclear membrane, validating the technical approach. Using transfected cells, we have been able to detect the activities of recombinant $InsP_3R$ channels, including wild-type and mutant constructs. Therefore, we have now established a mammalian cell system for proceeding with our work with the Bcl proteins. Two publications have resulted from this effort.

b. Second, we began to consider other molecular components of apoptosis pathways. Specifically, because intracellular $[Ca^{2+}]$ and intracellular Ca^{2+} stores may be involved in regulating apoptosis, and the $InsP_3R$ has been recently shown to be a substrate of caspase 3 (16), we examined the effects of caspase 3 on $InsP_3R$ channel activity. Caspase 3 is a key executioner caspase involved in apoptosis pathways (17). The type 1 $InsP_3$ receptor contains one consensus site for cleavage by caspase 3, and it was recently shown to be a substrate for caspase 3 (16). We hypothesized that cleavage by caspase 3 of the $InsP_3R$ may link the apoptosis pathway to Ca^{2+} signalling. We examined the effects of purified recombinant caspase 3 on the ion channel properties of the *Xenopus* type 1 $InsP_3R$. Caspase 3 was included in the pipette solution. Control patches were performed with the pipette solution lacking the enzyme, or containing a specific caspase 3 inhibitor peptide. We found that caspase 3 activates the $InsP_3R$ channel (Fig. 1,2). This effect does not require $InsP_3$, because inclusion of the $InsP_3$ competitive inhibitor heparin had no effect. The channels in the presence of heparin were not observed in control patches, or in patches with pipettes containing the capsase 3 inhibitor. This result represents the first observation

of channel activity of the $InsP_3R$ that does not require $InsP_3$ ligand. It suggests that activation of caspase 3 during apoptosis could induce a spontaneous Ca^{2+} leak into the cytoplasm from the endoplasmic reticulum.

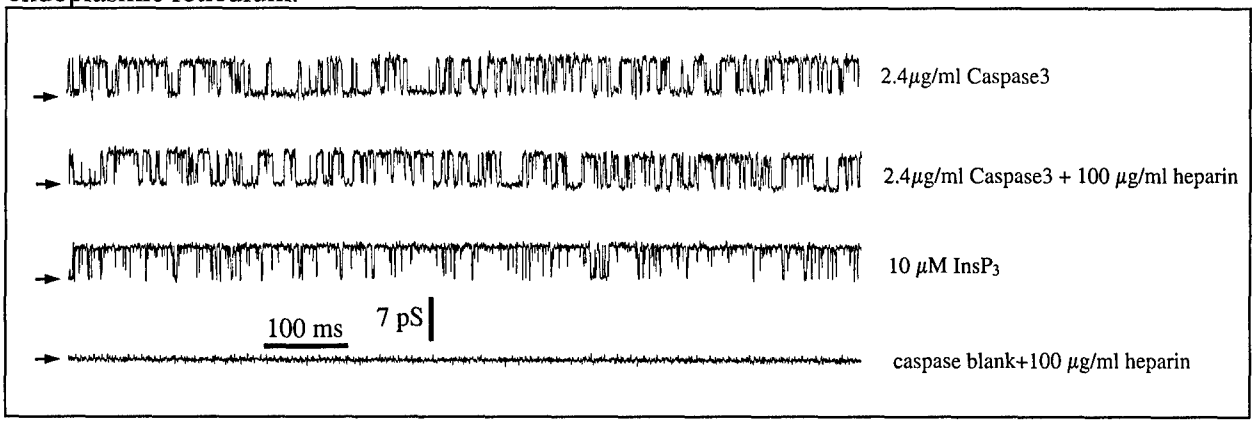
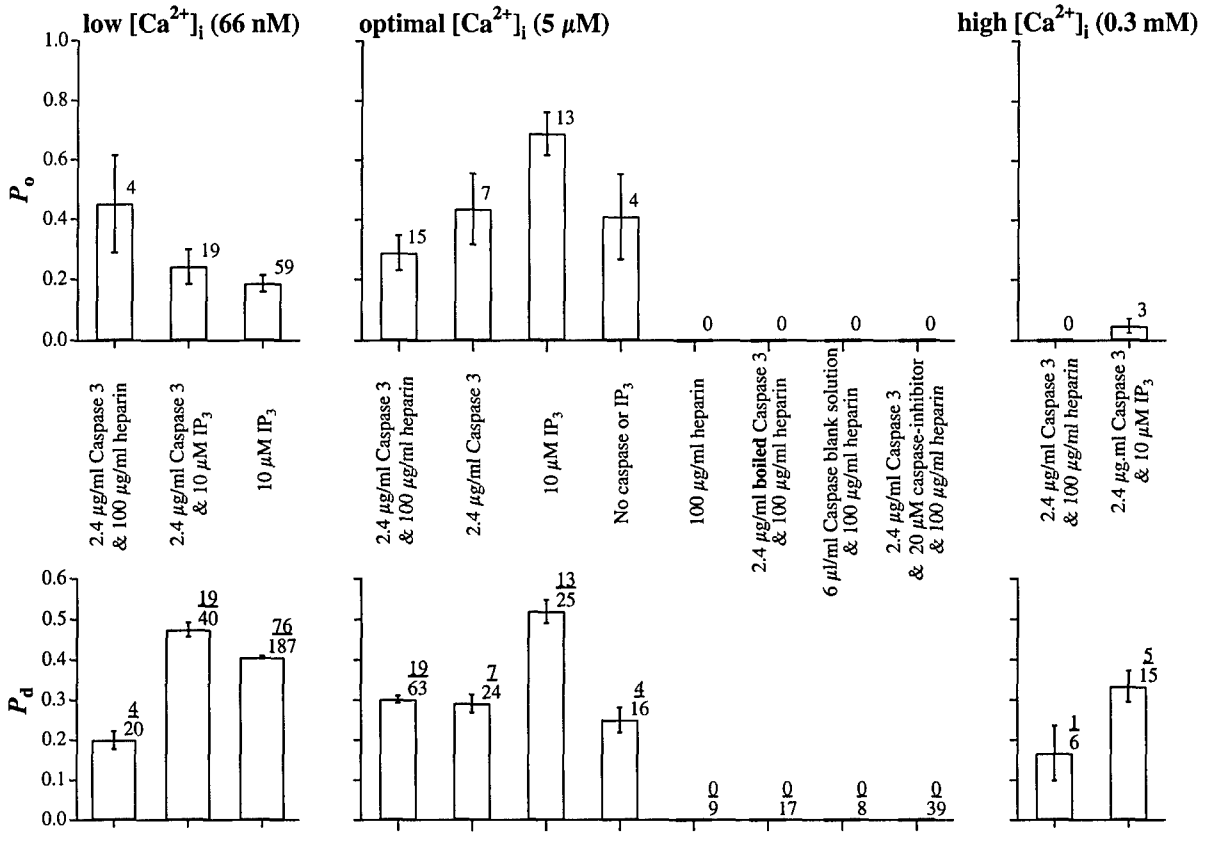


Fig. 1. Single channel current traces of *Xenpous* InP_3R type 1 activated by recombinant caspsase 3 in the absence (top trace) or presence (bottom trace) of the $InsP_3$ competitive inhibitor heparin, or by $InsP_3$ (third trace). Inactive caspase is without effect (last trace)

(The number of experiments used to calculate P_o is tabulated next to the bars)



(Tabulated next to the bars are: [number of experiments with $InsP_3R$ channel activity] / [total number of experiments performed])

Fig. 2. Summary of the channel open probability (P_o) or probability of detecting active channels in patches of membrane (P_d) under various conditions of cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ in the presence or absence of active or inactivated caspase 3 with or without $InsP_3$ or heparin.

Proposed studies during the final year

We are encouraged that we have been able to develop a mammalian system for the expression of recombinant endoplasmic reticulum-associated membrane proteins and subsequently isolate their nuclei and achieve giga-ohm seals. We will now utilize this system, employing transient transfection of Bcl-related cDNAs as we originally proposed in our application. We will also begin to undertake experiments outlined in Aim 3, again utilizing the new mammalian cell system with co-transfection of $InsP_3R$ cDNAs.

We will continue to pursue our observations related to the activation of Ca^{2+} release by caspase 3 to ensure productivity in this funding cycle while the bcl work progresses and develops into a productive approach. Specifically, we will utilize recombinant mammalian $InsP_3R$ to ask whether a similar effect occurs with the mammalian channels. This is particularly important, because only the type 1 isoform has the consensus caspase site, yet mammary epithelial cells may express other isoforms as well. Identification of isoform specificity will enable determination of important sequences in the $InsP_3R$ responsible for the caspase–induced leak. This knowledge may provide insights into potential theraputic targets. We will also examine the effects of other caspases to identify the specify from the protease perspective.

KEY RESEARCH ACCOMPLISHMENTS during the second granting period.

- Development of a mammalian cell system for expression of ER-localized recombinant proteins and patch clamp electrophysiology of their isolated nuclei.
- Identification of an effect of caspase-3 mediated cleavage of the $InsP_3R$ on its ion channel activity, inducing spontaneous Ca^{2+} channel activity.

REPORTABLE OUTCOMES

Boehning, D., S.K. Joseph, D.-O.D. Mak and J.K. Foskett. 2001. Single-channel recordings of recombinant inositol trisphosphate receptors in mammalian nuclear envelope. Biophysical J. (in press).

Boehning , D., D.-O.D. Mak, J.K. Foskett and S.K. Joseph. 2001. Molecular determinants of permeation and selectivity in inositol 1,4,5-trisphosphate receptor Ca^{2+} channels. J. Biol. Chem. **276**:13509-13512.

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